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Research paper

Downregulation of aldose reductase is responsible for developmental abnormalities of the silkworm purple quail-like mutant (*q-l*^{p})

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ABSTRACT

Aldose reductase (AR) is a rate-limiting enzyme in the polyol pathway and is also the key enzyme involved in diabetic complications. The silkworm purple quail-like mutant (*q-l*^{p}) exhibits pigmented dots on its epidermis. The *q-l*^{p} mutant also shows developmental abnormalities and decreased vitality. In this study, fat bodies from the *q-l*^{p} mutant and the wildtype 932VR strain were subjected to two-dimensional gel electrophoresis (2-DE) analysis, and the *Bombyx mori* AR (*BmAR*) protein was found to be significantly downregulated in the *q-l*^{p} mutant. The expression of *BmAR* at the mRNA level was also significantly downregulated, as verified through quantitative reverse transcription PCR (qRT-PCR). Knockdown of the expression of *BmAR* via RNAi resulted in a reduction of silkworm weight. The sorbitol level in *q-l*^{p} was significantly lower than in the wildtype. These results suggested that the *BmAR* gene is closely related to the development of the *q-l*^{p} mutant. Investigation of the cause of *BmAR* downregulation in the *q-l*^{p} mutant could contribute to revealing the function of *AR* in insects and offers a new method of identifying *AR* inhibitors for the treatment of diabetic complications.

1. Introduction

Aldose reductase (AR, EC: 1.1.1.21), belonging to the aldo-keto reductase superfamily, catalyzes the reduction of aldehydes or ketones to alcohol, with nicotinamide adenine dinucleotide phosphate (NADPH) serving as the coenzyme (Anil Kumar and Bhanuprakash Reddy, 2007). AR is the rate-limiting enzyme in the polyol pathway and exists in monomer form in many tissues. The molecular weight of AR is 30–40 kD, and its isoelectric point appears to be weakly acidic. The representative three-dimensional structure of AR contains eight α-helices surrounding a core of eight β-strands, all in parallel orientation, which constitutes an elliptic hydrophobic pocket. Tyr-48 and His-110 participate in acid-base catalysis (Anil Kumar and Bhanuprakash Reddy, 2007).

Researches for mammals have been relatively extensive, but rarely research in insects. In vivo of mammals, the majority of glucose is phosphorylated and provides energy for organisms through the glycolysis and tricarboxylic acid cycles. However, when AR is activated, the polyol metabolic pathway will in turn be activated, leading to sorbitol accumulation. Sorbitol shows strong polarity, making it difficult for sorbitol to penetrate the cell membrane. Thus, pathological changes will occur upon accumulation (Kador, 1990). Overexpression of AR also consumes large amounts of NADPH, which affects some physiological processes that require NADPH (Williamson et al., 1993). This is the pathological basis of diabetic complications, in which AR plays a key role (Lefrançoismartinez et al., 2004; Maritim and Sanders, 2003). For diabetics, hyperglycemia is the activating factor for AR (Gu et al., 2010; Wang et al., 2004). To reduce the damage from diabetic complications, AR activity should be inhibited to prevent sorbitol accumulation and thus ameliorate the damage to the organism from diabetic complications (Oates, 2010; Schemmel et al., 2010). However, the exact mechanism leading to diabetic complications mediated by AR is still unclear. In clinical trials, AR inhibitors continue to exhibit many side effects, such as lack of efficacy, skin sensitivity, and liver toxicity.

The fat body is one of the largest tissues of *Bombyx mori*, with components including fat, glycogen, protein and vitamins. The fat body

Abbreviation: q-l^{p}, purple quail-like; AR, aldose reductase; BmAR, *Bombyx mori* aldose reductase; 2-DE, two-dimensional gel electrophoresis; qRT-PCR, quantitative reverse transcription PCR; NADPH, nicotinamide adenine dinucleotide phosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; NCBI, National Center of Biotechnology Information; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RNAi, RNA interference; EGFP, enhanced green fluorescence protein; dsRNA, double-stranded RNA; BLAST, Basic Local Alignment Search Tool; RPKM, reads per kb per million reads; G6PI, glucose-6-phosphate isomerase; G6PD, glucose-6-phosphate dehydrogenase; UGTs, UDP-glycosyltransferases; GO, gene ontology

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exhibits important physiological functions, not only storing a large number of nutrients but also serving as an important site of biosynthesis and metabolism (Arrese and Soulages, 2010). Additionally, the fat body is an important immune organ and takes part in the detoxification mechanisms of many molecules (Enayati et al., 2005). The silkworm purple quail-like mutant (*q-l*^{p}) exhibits pigmented dots on its epidermis. In addition, *q-l*^{p} shows developmental abnormalities and decreased vitality. Therefore, we asked whether there is a relationship between the decreased vitality of the *q-l*^{p} mutant and decreased fat body metabolism. Hence, the fat body of the *q-l*^{p} mutant was used as material for two-dimensional gel electrophoresis (2-DE) analysis, with the fat body of the wildtype 932VR strain serving as the control. The results showed that the expression level of *BmAR* in the *q-l*^{p} mutant was significantly lower than in the wildtype, which remained the same at the RNA level, as verified by quantitative reverse transcription PCR (qRT-PCR). RNAi experiments and determination of sorbitol concentrations indicated that downregulation of *BmAR* may be related to the developmental abnormality phenotype of the *q-l*^{p} mutant (Zhao et al., 2014). These results provide experimental evidences related to studying the functions of *AR* in the *q-l*^{p} mutant.

2. Materials and methods

2.1. Silkworm rearing and tissue isolation

The *q-l*^{p} mutant and wildtype 932VR silkworm strains were supplied by the Sericulture Research Institute, Chinese Academy of Agricultural Sciences (Zhenjiang, China). The larvae were fed fresh mulberry leaves under standard conditions: 25 ± 2 °C, 12-h light/12-h dark photoperiod and 65 ± 5% relative humidity. When the silkworms had developed to the appropriate stage, tissues were dissected and subsequently stored at −80 °C.

2.2. Protein extraction and 2-DE

Fat bodies collected on the 3rd day of the 5th instar were ground to a powder using liquid nitrogen. The extracted liquid (8 mol/L urea, 2% chaps, 0.12% destreak reagent) was then used to extract total protein. The Bradford method (Bradford, 1976) was employed to quantify the protein concentration, after which the protein samples were stored at −20 °C.

Based on the concentration of total protein, 150 μg of protein was subjected to isoelectric focusing electrophoresis using an IPG gel strip with a pH range of 3–10. Thereafter, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 12.5% separation gels. After silver staining, differentially expressed protein spots were analyzed using ImageMaster™ 2D Platinum 6.0 software (GE Healthcare). Finally, the differentially expressed protein spots were sent to BGI (Beijing Genomics Institute) for mass spectrometry analysis using MALDL-TOF/TOF-MS method, and bioinformatics analysis was performed using the silkDB (http://silkworm.swu.edu.cn/silkdb/) database and NCBI (National Center of Biotechnology Information) (https://www.ncbi.nlm.nih.gov/) databases.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from all tissues using RNAiso Plus (TaKaRa, China) and then dissolved in RNase-free water. The concentration of total RNA was determined using a NANODROP1000 microspectrophotometer (Thermo, USA) after treatment with DNase. Then, cDNA was synthesized using PrimeScript™ RT Master Mix (TaKaRa, China), and the concentration of cDNA was determined using a NANODROP1000 microspectrophotometer (Thermo, USA).

2.4. Quantitative reverse transcription PCR

The cDNA samples were diluted to 50 ng/μL and used as the template for qRT-PCR. Each 20-μL reaction included 1 μL of primers (10 μM, Forward primer: AAACGCCTCAAGGAGTTCTG, Reverse primer: ATCACGATGTTCCCGCTGTC), 1 μL of cDNA, 10 μL of 2× SYBR 1 Premix Ex Taq™ (Tli RNaseH Plus) (TaKaRa, China) and 8 μL of ddH_2O. After a rapid centrifugation step, qRT-PCR was performed using a LightCycler 96 real-time PCR system (Roche, Switzerland), with the following three-step reaction program: 10 min of pre-degeneration, followed by 45 cycles of 95 °C for 10 s, 55 °C for 10 s and 72 °C for 10 s, and then a final melting step. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), employing glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, *BGIBMGA007490*, forward primer: TTCATGCCACAACTGCTACA, reverse primer: AGTCAGC TTGCCATTAAGAG) as the reference gene. Melting analysis was performed to guarantee the quality of the qRT-PCR results.

2.5. RNA interference

Reverse transcription PCR was performed to obtain the template for in vitro transcription using a pair of primers including the T7 promoter (BmAR_RNAi_F: GAAATTAATACGACTCACTATACTGTGGAACACGTTT CATCGC, BmAR_RNAi_R: GAAATTAATACGACTCACTATAGACTCCGA TGCTCTTGACCAG). The *EGFP* gene served as the control (primer sequences: EGFP_RNAi_F: GAAATTAATACGACTCACTATAACTGCATGTA CCACGAGTCCA, EGFP_RNAi_R: GAAATTAATACGACTCACTATAGTAC ATGCTCACGTCGCCCTT). DsRNA was obtained via in vitro transcription (Quan et al., 2002) and then diluted to 2 μg/μL after treatment with DNase and RNase. When silkworms of the 932VR strain had developed to the newly exuviated stage of the 4th instar, two groups of 20 silkworms each, with the same size and consistent developmental times, were selected for RNAi. One group was injected with dsRNA corresponding to *BmAR* and the other with dsRNA corresponding to *EGFP*, at a dosage of 2 μL in each silkworm. Then, the average weights of these two groups as well as the *q-l*^{p} mutant that injected with deionized water were determined to analyze the effect of the *BmAR* gene on silkworm development. Except 6 silkworms for qRT-PCR, the remain 14 silkworms were used for weight statistics.

2.6. Determination of sorbitol levels in silkworm blood

Sorbitol can react with cupric hydroxide to form a blue complex in alkaline solution that shows maximum absorbance at a wavelength of 655 nm, and there is a linear relationship between the absorbance and the sorbitol concentration (Liang, 2007). Thus, spectrophotometry was employed for the determination of sorbitol levels in this study. First, a standard curve was constructed with a standard sorbitol solution. Then, the blood of wildtype 932VR and *q-l*^{p} mutant silkworms in the 3rd day of 5th instar was isolated. The treatments of blood were based on the instructions for sorbitol content kit produced by Solarbio company (http://www.solarbio.com). After the addition of 0.5 mL of deionized water to a 0.5 mL blood sample, the diluted sample was treated in a water bath at 95 °C for 10 min. The supernatant was then diluted approximately 5-fold after centrifugation, and the diluted sample could be used for the determination of sorbitol levels. The assay procedure was as same as for the determination of standard sorbitol. The blood sample was diluted approximately 10-fold in total throughout the determination process.

2.7. Construction of the evolutionary tree

The amino acid sequence of *BmAR* was used for BLAST (Basic Local Alignment Search Tool) analysis in the NCBI database. The identified sequences with high homology were employed to a construct evolutionary tree with a MEGA5 software. Default parameters of software